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Dynamic change of protein polypeptide of *Ginkgo biloba* seed during germination

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Abstract: The dynamic changes of protein polypeptide in endosperms of *Gingkgo biloba* seeds during seed germination were studied by SDS-PAGE and two-dimensional gel electrophoresis (2-DE). The results showed that 80 kinds of protein spots in endosperms of *Gingkgo biloba* were clear observed in the 2-DE spectrum. Protein molecular weights were in the range of 26–52kD, and their isoelectric points were in the range of 5.8–7.8. In the course of seed germination, 13 kinds of proteins were degraded, and 13 kinds of proteins were synthesized; 7 kinds of proteins with different molecular weights and isoelectric points of 35kD/pI6.8, 31kD/pI6.8, 29kD/pI6.8, 33kD/pI6.6, 33kD/pI 6.4, 34kD/ pI7.7 and 31kD/pI7.7 were identified primarily as vegetative storage proteins (VSPs).

Keywords: *Ginkgo biloba* seed; Germination; Protein; Electrophoresis

Introduction

Vegetative Storage Protein (VSP) was the main form of reserving nitrogen in many plants (Fu 1985). At present the study of VSP is very popular, and there are many correlative reports in global field. The VSP of *Gingko biloba* were found firstly by Fu Jiarui (1985). Subsequently, Peng Fangren and Guo Juan did further studies on the localization and qualitative of VSP of *Ginkgo biloba*, and found some change rules of VSP (Peng *et al.* 2001). However, there was not any report about VSP in Gingko seed. The studies on changes of VSP of seeds during seed germination mainly concentrated on crops (Santos and Ferreira. 1998; Shatter 1997; Xu *et al*.1992; Liu *et al*. 2001; Wang *et al*. 1992; Lin *et al*.1995), but less attention is paid to the seeds of woody plants.

In this paper, the dynamic change of Protein Polypeptide in the molecule level by electrophoresis was studied. Storage Proteins, as the one of main compositions of storage substance in seed, offered the nutrient substance and energy for the germination of seed and the growth of seedling. In general, its degradation was divided into three steps as follows: (1) storage proteins were modified; (2) the storage proteins of high molecule weight were degraded into low molecule protein (peptide), (3) the latter was degraded into amino acids. The character of protein composition and the dynamic change of protein polypeptide of endosperm in Gingko seed were studied by SDS-PAGE and 2-DE, and the composition of key proteins was primarily identified, and their change rule was ascertained.

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Materials and methods

Experiment materials

Ginkgo biloba seeds came from Taixing City of Jiangsu Province, Chian and were stored in wet sand. The mixture of seeds and wet sand with the volume ratio of 1:10–1:15 was piled up in the barrel with some holes under the bottom on the ventilated and cool place and were churned and watered every two weeks for the after ripening. After being stored for 2–3 months, the seeds were used for germination in March 2003. The endosperms of *Gingkgo biloba* seeds were separately taken at the 3rd, 6th, 9th, 13th, 18th and 22nd day of germination. The samples were cleaned and seed capsule was removed. The embryo, endosperm and buds were separated from seeds with the scalpel and were placed in the airproof plastic bags and stored at -70°C

Experiment methods

Extraction of protein

Five granules of endosperms were taken out from refrigerator (-70°C) and pestled in the pre-cold mortar on the condition of ice. After then, they were transferred into the Eppendorf tube and was extracted by 2 double volume of the sample buffer containing 2% of SDS, 5% of β-Mercaptoethano, 10% of glycerin, 0.01 mol·L^{-1} Tris-HCl of pH8.0 for 1 h at 4°C, and then centrifuged at 15000 r·min^{-1} for 10 min. The upper extraction solution was received. Proteins within the extraction were precipitated with the 2 double volume of cold acet for 3 h at -20°C, and then centrifuged with the same centrifugal condition. The upper solution was discarded and the deposits were watered twice with cold acet. The deposits were dried at 30°C, and dry powders were collected for the analysis of protein composition of SDS-PAGE and 2-DE.

SDS-PAGE

The dry powder of 5 mg was dissolved by the sample buffer of 200 μL in the Eppendorf tube of 1.5 mL at the usual temperature,

and centrifuged at 15000 r·min^{-1} for 10 min at 4°C. The upper extraction solution was received. The extraction solution of 10μL was loaded in the slot (about 250-μg protein). The SDS-PAGE was performed respectively in the concentrated gel of 5% for 1 h at 20 mA, and in the separated gel of 12% at 25 mA until it was finished. The electrode buffer was the complex of the Tris-glycin, 0.1% of SDS, 0.384 mol·L^{-1} of glycin and 0.05 mol·L^{-1}of Tris of pH8.3. The gel was stained with 0.25%of the solution of Commassie Brilliant Blue R-250, acetum and carbinol for 4 h in the water of 40°C, then discolored with the acetum- carbinol-water solution, and photos were taken. The apparatus was mainly DYY-12 electrophoresis machine.

Two-dimensional gel electrophoresis (2-DE)

IEF-PAGE: Four 2-mm-diameter glass tubes with no bottom were bound together and placed in the measuring cylinder of 50 mL, and filled with the gel solution, then water was filled to make the gel solution enter into the tubes. The gel was covered with 8-mol·L^{-1} urea of 17μL on the surface, and polymerized at 20–35°C and the gel strip was taken out to insert in the slot of 2-DE. The electrode buffers were 1000-mL H_3PO_4 (0.01 mol·L^{-1}) in the down-slot and 500-mL NaOH (0.02 mol·L^{-1}) in the up-slot. Voltage was designed at 200v for 15 min, 300v for 30 min, and 400v for 30 min. Dry powder of 5 mg was transferred into a 1.5-mL Eppendorf tube, and dissolved with the fissile solution which contained 9.0-mol·L^{-1} urea, 2% of NP-40, 1.6% of Ampholine of pH5–8, 0.4% Ampholine of pH3– pH 9.5, and 2% of DTT at usual temperature, then centrifuged at 15000r·min^{-1} for 10 min at 4°C. The upper solution was the sample of protein. The 50-μL protein sample was loaded in each tube. The 25-μL overlayed solution containing 8-mol·L^{-1} urea, 0.8% of Ampholine pH5–8 and 0.2% of Ampholine of pH 3–9.5 were filled on the sample. The usual voltage was designed as 600v for 7 h, 800v for 8 h and 1000v for 1 h. When the IEF-PAGE ended, the gel strip was peeled off and fixed with TCA of 50%(v/v) for 30 min, and rinsed with deionized water three times, and equilibrated for 3–5 min in the equilibration buffer with 10% of glycerin, 5% of β-Mercaptoethano, 2.3% of SDS and 0.625-mol·L^{-1} Tris-HCl, and then equilibrated again for 30 min in fresh one. Thus the gel strip can be used on SDS-PAGE.

SDS-PAGE: The system was the same to the above described one.

PI analysis: The method of pickling gel was used to measure the PI. The gel strip of the IEF-PAGE was taken and rinsed with deionized water three times, and cut into the fraction of 1cm along the gel strip to soak in 2-mL deionized water at 4°C for a whole night, and the PI gradient of gel strip was obtained by using the PH-meter.

Results and discussion

Morphology and shape of Gingko seed during seed germination

Gingko seed was made up of yellow exopleura, white mesosperm, brown endotesta, endosperm and embryo. Gingko seeds belong to the kind of seeds with high content of water. It was reported that the water content of *Ginkgo biloba* seed was 45%–50% of whole seed weight, and its thousand-grain-weight was 3 000–3 500 g (Yang *et al*. 2000). The *Ginkgo biloba* seed with thousand-grain-weight of 3 139.28g could not germinate until it finished afterripening (Cao *et al*.1995, 2002).

The morphology of Gingko seed during seed germination was described in the Table 1. When the embryo length was amount to 2.4 cm, the embryo stopped growing, and the bud of seed began to sprout. When the bud reached 4.3 cm nearly, new leaves began to photosynthesize by themselves. The morphologies and inclusions of seed during germination were described in Table 1.

Table 1. Seed morphology of Gingko seed during seed germination

Germination days (d)	Embryo length (cm)	Bud length (cm)	Seed morphology
0	0.8	——	flaxen endosperm, without cavity
3	1.6	——	flaxen endosperm, without cavity ,with hatch in the endopleura
6	2.4	——	endosperm kelly, radicel breaking through endopleura to germinate
9	2.4	0.7	endosperm kelly, bud growing
13	2.4	2.4	endosperm Kelly shrinking
18	2.4	3.6	endosperm kelly shrinking and crumpling
22	2.4	4.3	Endosperm kelly shrinking and crumpling

Dynamic change of endosperm protein of Gingko seed in SDS-PAGE

The endosperm protein of *Ginkgo biloba* seed mainly existed in the B and C section according to SDS-PAGE spectrum as described in Fig. 1. During seed germination, proteins with molecular weights of 85kD, 79kD, 75kD, 68kD, 65kD, 58kD, 31kD, 26kD, 28kD, 22kD, 20kD, 18kD, 16kD, 17kD, 15kD, 14kD and 13kD were primarily considered to exist in the endosperm of Ginkgo seed universally. The dynamic changes of protein polypeptide were described in the form of the strap concentration and the appearance and disappearance of strap on the spectrum.

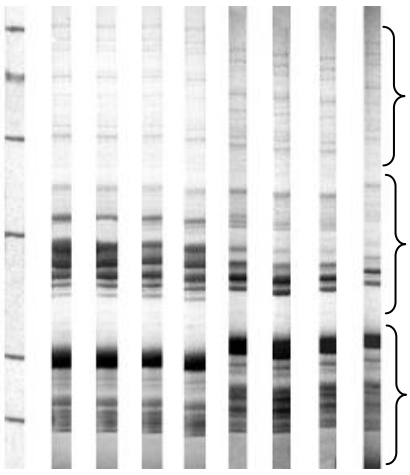

Fig. 1 SDS-PAGE of endosperm of *Ginkgo biloba* seed

1. Seed dormancy, 2. Embryo 0–1cm, 3. Embryo 1–2cm, 4. Embryo 2–3cm, 5. Sprout 0–1cm, 6. Sprout 2–3cm, 7. Sprout 3–4cm, 8. Sprout 4–5cm

It was observed that, in the SDS-PAGE spectrum, the dynamic changes of proteins with molecular weights of 85kD, 77kD, 50kD,

39kD, 34kD, 33kD, 28kD, 26kD, 24kD, 23kD, 17kD, 16kD and 13kD were obvious as described in Fig. 2, especially the dynamic changes of proteins in B section. During seed germination, some proteins with molecular weights of 85kD, 50kD, 34kD, 30kD, 28kD,and 23kD disappeared, and some proteins with molecular weights of 77kD, 36kD, 33kD and 13kD were synthesized, in addition, the color of some proteins with molecular weights of 39kD, 26kD, 24kD, 17kD and 16kD changed from deep to shallow till disappeared in the end. These disappearance proteins were decomposed and transformed into all kinds of amino acid and other proteins to offer the nutrition for the growth of embryo and bud. The new synthetic proteins maybe were: (1) decomposed products of storage proteins. (2) enzyme-proteins were synthesized first and disappeared in the end of germination.

There were about 150 kinds of protein spots in the spectrum of 2-DE, which were described as Fig. 3, and the molecular weights of these protein were in the range of 97–13kD, and their isoelectric points were in the range of 5.3–7.8. The abundance of protein polypeptide of 2-DE was more than that of SDS-PAGE. Among these protein spots, about 80 kinds of protein spots could be clearly observed, especially protein spots whose molecular weights were in the range of 26–52kD and whose isoelectric points were in the range of 5.8-7.8 were the most and stained deeply. The results showed that the protein content of endosperm of *Ginkgo biloba* seed was abundant.

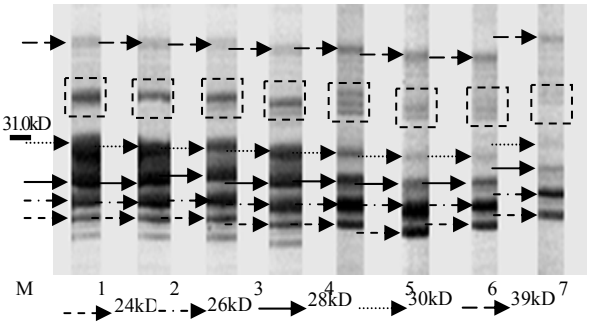

Fig. 2 SDS-PAGE of endosperm of *Ginkgo biloba* seed in B section
1. Seed dormancy, 2. Embryo 0–1cm, 3. Embryo 1–2cm, 4. Embryo 2–3cm, 5. Sprout 0–1cm, 6. Sprout 2–3cm, 7. Sprout 3–4cm, 8. Sprout 4–5cm.

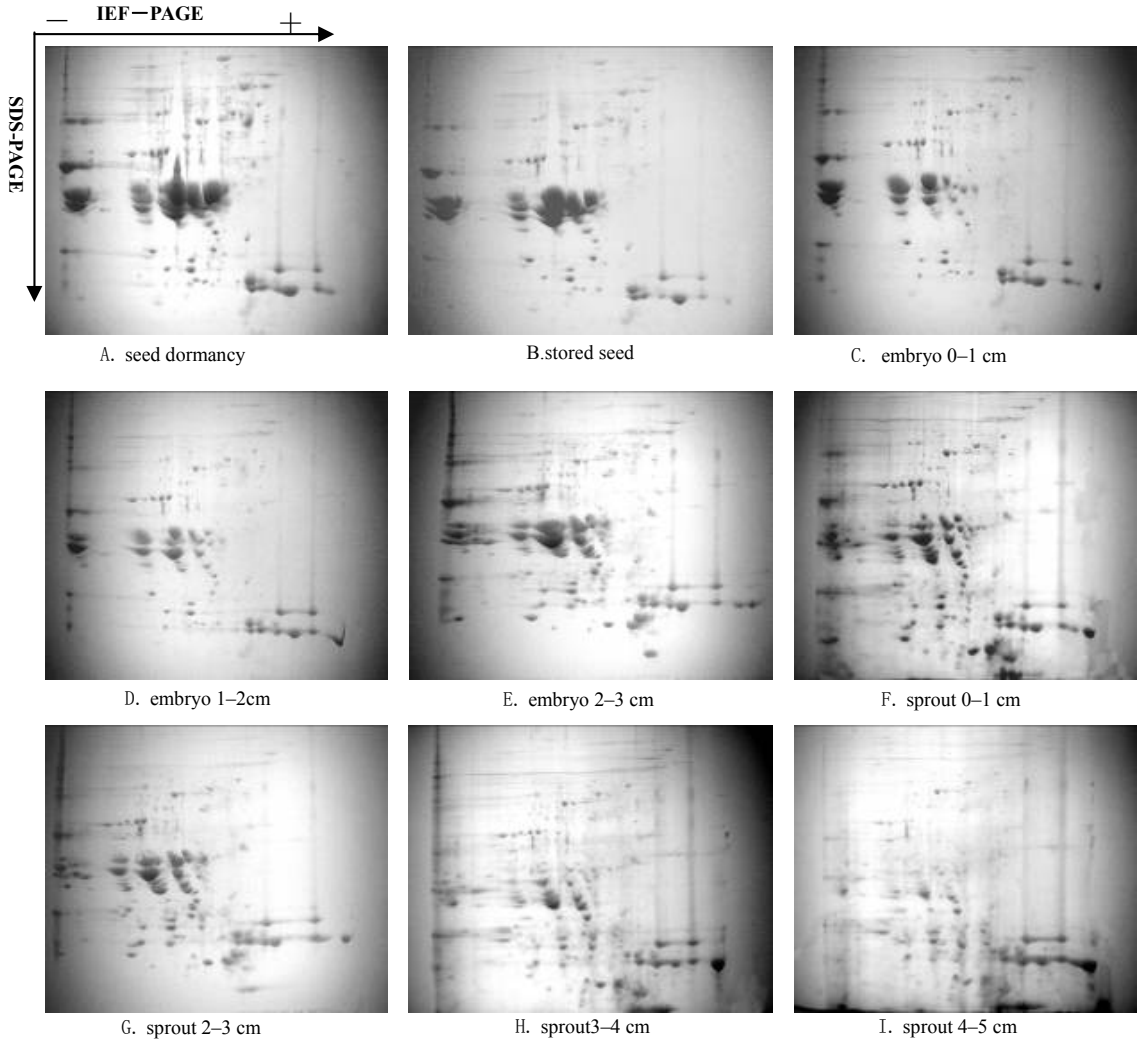

Fig. 3 2D SDS-PAGE of embryo of *Gingko biloba L.* seeds in the different germination

Dynamic change of endosperm protein of Gingko seed in 2-DE

The degraded proteins with different molecular weights and isoelectric points were the proteins of 31kD/pI6.8, 31kD/pI7.1, 31kD/pI7.7, 33kD/pI6.6, 33kD/pI7.1, 34kD/pI7.7, 36kD/pI7.1, 42kD/pI7.8, 46kD/pI7.2, 46kD/pI7.0, 46kD/pI6.9, 85kD and 50kD during germination. The seed germination needed nutrition and energy. Therefore proteins were degraded and transformed into all kinds of amino acid and other proteins to provide nutrition for the growth of embryo and buds.

New synthetic proteins with different molecular weinghts and isoelectric points included proteins of 14kD/pI6.3, 15kD/pI6.0, 15kD/pI6.1, 16kD/pI6.2, 17kD/pI6.6, 31kD/pI6.7, 31kD/pI7.0, 31kD/pI6.3, 33kD/pI6.4, 34kD/pI7.4, 35kD/pI6.2, 35kD/pI6.3, 35kD/pI7.5, which degraded and disappeared gradually with germination. The possible reasons why the new proteins were synthesized were as follows: (1) the above proteins possibly were the middle products of degraded proteins. The storage proteins were decomposed into polypeptides or dipeptides by protease first, and then were decomposed to amino acid by endopeptidase; (2) the above proteins maybe were enzyme proteins. The decomposition of proteins, starch and fat in seed could be finished by enzymes. So lots of enzyme proteins must be synthesized in the process; (3) the above proteins maybe were amides, lots of ammonia were harmful to plants, so the accumulaion was necessary in the form of amides.

Identification of Storage Proteins

Based on the analysis of spectrum of SDS-PAGE and 2-DE, the 7 kinds of proteins with different molecular weights and isoelectric points of 35kD/pI6.8, 31kD/pI 6.8, 29kD/pI6.8, 33kD/pI6.6, 33kD/pI6.4, 34kD/pI7.7 and 31kD/pI7.7 were identified as vegetative storage proteins. These protein spots were big when *Ginkgo biloba* seed didn't germinate, then became small gradually with germination until disappeared in the end. These trends consist with the criterion of VSPs.

Conclusions

In this experiment, *Ginkgo biloba* seeds during seed germination were studied with the method of SDS-PAGE and 2-DE. Both the morphology and the inclusions took place some changes, especially endosperm proteins. During seed germination, some storage substances were decomposed to all kinds of acid amino and small molecular proteins to provide nutrition for the embryo and buds growing. The endosperm proteins (35kD/pI6.8, 31kD/pI 6.8, 29kD/pI6.8, 33kD/pI6.6, 33kD/pI6.4, 34kD/pI7.7 and 31kD/pI7.7) were primarily identified as vegetative storage proteins, according to a series of changes of proteins in SDS-PAGE and 2-DE.

Discussion

SDS and β-Mercaptoethano were applied during protein sample preparation, which made protein denaturalize completely and depolymerize to subunit or single peptide chain, so the result analyzed was only the molecular weight of subunit and single peptide chain.

In the paper, storage proteins were ascertained on the basis of the criterion of VSP. Firstly storage protein content in soluble proteins was more. Secondly the seasonal change rules of protein straps were obvious. That is to say, the protein straps were clear observed first then disappeared gradually in the sprctrum (Tian 2002). However, whether these proteins really were vegetative storage proteins or not, it needed to be studied further with western blotting (Yu *et al.* 2004).

Some protein gels might be sensitive to silver staining and others were sensitive to Coomassie blue. When the volume of sample was little, silver staining was better than Coomassie blue, because the protein content was enough, we used Coomassie blue to dye the gel.

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